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DISCLAIMER

ABSTRACT

Under this USDOE-NETL contract, the bacterium *Pseudomonas fluorescens* strain CL0145A is being developed as a biocontrol agent of both North American zebra mussel species, *Dreissena polymorpha* and *D. bugensis*. The experimental data presented in this report indicate that the choice of culturing method is critically important in order for *P. fluorescens*-CL0145A cells to achieve high mortalities against both mussel species. The potential market for *P. fluorescens*-CL0145A as a biocontrol agent will obviously be much more extensive if, as a commercial product, it is efficacious against both mussel species. Tests will soon be conducted at the Rochester Gas & Electric research trailer to develop treatment dosages designed to achieve high mussel kill against both mussel species in service water at this power plant. The unique nature of our *P. fluorescens* strain CL0145A as an effective control agent was further verified in concurrent tests with three other strains of *P. fluorescen*s, all of which have been partially or totally DNA-sequenced. These tests indicated that strain CL0145A was the only strain capable of inflicting high levels of mortality on zebra mussels. This is a critically important research result that will be highlighted in a DOE - Joint Genome Institute proposal to have *P. fluorescens* strain CL0145A sequenced (to be submitted in 2006).

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EXECUTIVE SUMMARY

Use of the bacterium *Pseudomonas fluorescens*-CL0145A represents a potential alternative to the current use of polluting biocides for control of zebra mussel infestations in water pipes. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent. This is to be achieved by developing economical methods to produce and formulate the bacterial cells in order that they will prove to have good product shelf-life, be environmentally safe, and be highly effective in killing zebra mussels in power plant water pipes. During the six month period reported herein, research efforts focused primarily on the following topics:

- Cell toxicity: During our 2004 testing program, cells of *P. fluorescens* strain CL0145A that were harvested from a traditional growth medium consistently achieved relatively low levels of mortality against one of the two species of zebra mussels in North America, *Dreissena bugensis* (also known as the quagga mussel). Quagga mussel mortality was often an order of magnitude lower than that of zebra mussels (*D. polymorpha*). If *P. fluorescens*-CL0145A is ever to become a commercially available biocontrol agent, it is essential that it be efficacious against both zebra and quagga mussels. The data presented in this report indicate that the choice of a bacterial cell culturing method is critically important in order for *P. fluorescens*-CL0145A to achieve high mortalities against both zebra and quagga mussels when treated in pipes under flow-through conditions.
- Unique nature of CL0145A: There are already three strains of *P. fluorescens* whose entire genomes have been (or are currently being) sequenced, i.e., *Pf*-5, *Pf*0-1, and *Pf*-SBW25. A proposal submitted to the DOE - Joint Genome Institute by our laboratory in February 2005 was not approved. This was largely because there was lack of evidence that our strain CL0145A was unique in its ability to cause significant mortality among zebra mussels. Efficacy testing of these three strains has now confirmed that our strain CL0145A is unique in its effectiveness as a highly toxic zebra mussel control agent. These results are very significant for our project since they further underscore the importance of sequencing *P. fluorescens*-*C*L0145A. Whole-genome sequencing of *P. fluorescens*-*C*L0145A would significantly increase the chances of its successful commercial development as a microbial pesticide for zebra mussel control. Following identification of the genes of interest, toxin production could be increased through site-directed mutagenesis and metabolic engineering, leading to a more effective commercial product. In addition, identifying the toxin would accelerate progress in a variety of research activities required for commercialization, such as: protocol improvements relating to bacterial fermentation, downstream processing, formulation, and product storage. At each step in these latter activities, the quantity of toxin could be monitored by relatively quick chemical analysis rather than by the more time-consuming, traditional, labor-intensive approach of performing bioassays against zebra mussels. Although we have already made significant progress in devising improved protocols and culturing media for producing toxic cells, due to the low cost of currently used chemical pesticides, it will be very difficult to successfully commercialize *P. fluorescens*-CL0145A unless production costs can be considerably reduced. We believe that the primary avenue to achieve this goal is through genetic enhancement. That is why it is so critically important to have the whole genome of *P. fluorescens*-CL0145A sequenced.

INTRODUCTION

Coal-fired power plants within North America need an effective, economical, and non-polluting technique for managing infestations of the zebra mussels (*Dreissena polymorpha* and *D. bugensis*) within their facilities. Due to a lack of options, many facilities have relied on the use of broad-spectrum, chemical biocides for control of these freshwater mussels. Biocide treatments, such as continuous chlorination for three weeks, however, are widely regarded as environmentally unacceptable because they can result in the formation of potentially carcinogenic substances. Use of the bacterium *Pseudomonas fluorescens* strain CL0145A represents a potential alternative to the use of polluting biocide treatments and is the leading candidate in the world for the biological control of these macrofouling mussels. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent. This is to be achieved by developing economical methods of producing and formulating the bacterial cells in order that they will prove to have good product shelf-life, be environmentally safe, and be highly effective in killing these mussels in infested power plant water pipes. During the six month period reported herein, research efforts focused on the following two project activities:

1. DETERMINING THE EFFICACY OF CL0145A GROWN IN AN EXPERIMENTAL MEDIUM AGAINST QUAGGA AND ZEBRA MUSSELS UNDER FLOW THROUGH CONDITIONS.

The more toxic each *P. fluorescens*-CL0145A cell is, the fewer cells that would be required in an actual zebra mussel treatment. The need to use fewer cells should consequently result in lower treatment costs. Laboratory experiments to define the key nutrients and culture conditions required to produce more toxin per bacterial cell had commenced during the previous reporting period and had resulted in the development of an experimental growth medium which showed promise in effectively controlling both species of zebra mussels present in North America: *D. polymorpha* (the common zebra mussel) and *D. bugensis* (the quagga mussel). During 2004, bacterial treatments using cells cultured in a traditional growth medium displayed difficulty producing high levels of mortality against quagga mussels. Quagga mussel mortality was often an order of magnitude lower than that of the common zebra mussel. If *P. fluorescens*-CL0145A is ever to become a commercially viable biocontrol agent, it is essential that it be highly efficacious against both the zebra mussel and the quagga mussel, as over time quagga mussel populations have been slowly replacing zebra mussel populations throughout their North American range.

At the Rochester Gas & Electric (RG&E) Russell Power Station, where we conduct our field trials, approximately 95% of the fouling mussels are quagga mussels. Within power plants, mussels typically reside in pipes and other locations where water is flowing and avoid static areas. Traditional laboratory testing is centered around experiments in aerated jars because they are easily managed, can evaluate many variables at once, and are much less time consuming to conduct. Mussels in power plants, however, are under flow-through conditions in pipes, so it is important to show that *P. fluorescens*-CL0145A cells, when harvested from experimental growth media, are capable of killing zebra and quagga mussels equally well under more realistic, i.e., flow-through, pipe conditions. Toward this end, experiments were conducted under laboratory conditions against both mussel species under flow-through conditions in pipes. These tests used CL0145A cells produced with a new experimental culturing medium and successfully achieved ca. 90% kill of both mussel species.

2. CONFIRMING THE UNIQUE NATURE OF *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A.

Herein we report comparative test data examining the relative toxicity of three sequenced strains of *P. fluorescens* (*Pf*-5, *Pf*0-1, and *Pf*-SBW25) to zebra mussels. These data indicate that strain CL0145A still retains its unique position as the only known *P. fluorescens* strain that is highly toxic to zebra mussels.

Why were these tests with the three sequenced strains of *P. fluorescens* conducted? Whole-genome sequencing of *P. fluorescens*-CL0145A would significantly increase the chances of its successful commercial development as a microbial pesticide for zebra mussel control. Once the genome of CL0145A is sequenced, efforts would focus on identifying the gene(s) involved in the production and regulation of the molecular product of interest, i.e., the zebra mussel-killing toxin produced by *P. fluorescens*-CL0145A, thus providing insight into the identity and nature of the toxin itself.

On an international basis, the above-mentioned three strains of *P. fluorescens* are the only strains whose entire genomes have been (or are currently being) sequenced. A proposal for the whole genome sequencing of our CL0145A strain of *P. fluorescens* was submitted to the DOE-JGI-CSP (DOE Joint Genome Institute – Community Sequencing Program) by our laboratory in February 2005. It narrowly missed approval, primarily because the reviewers indicated there was a lack of evidence that strain CL0145A was unique in its ability to cause high mortality against zebra mussels. The reviewers questioned the need to sequence our CL0145A strain considering that one of the existing above-mentioned three sequenced strains might already be highly toxic to zebra mussels. Thus, we chose to perform these comparative tests reported herein examining the relative toxicity of these three sequenced strains of *P. fluorescens*.

EXPERIMENTAL

The following is an overview of the materials and methods used in tests completed in activities #1 and #2.

1. DETERMINING THE EFFICACY OF CL0145A GROWN IN AN EXPERIMENTAL MEDIUM AGAINST QUAGGA AND ZEBRA MUSSELS UNDER FLOW THROUGH CONDITIONS.

The following is a general outline of the methodology employed in these culturing tests.

- Shaken seed cultures: 250-ml Erlenmeyer flasks containing 25 ml of buffered tryptic soy broth (bTSB) were inoculated with 0.4 ml of stock culture and shaken at 200 rpm at 26±1ºC for 24 hr.
- Shaken flask cultures: 0.35 ml from the bTSB 24-hr shaken seed cultures was used to inoculate each of many replicate flasks containing the experimental medium. Flasks were shaken at 200 rpm at 26±1ºC for 24 hr.
- Static flask cultures: 1.00 ml from the bTSB 24-hr shaken seed culture was used to inoculate each of many replicate flasks containing 100-ml bTSB. Flasks were cultured statically at 26±1°C for 72 hr.
- Production of cell fraction (CF): The final whole culture (FWC) from each type of growth medium flask was centrifuged together to produce a common pellet for each medium type. FWC were centrifuged (60 min at 1449 x g) in 500-ml batches in 700 ml centrifuge bottles, and cell pellets were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \bullet 6H_2O$ in deionized water).

- Preparation of CF: Mean dry bacterial cell mass/ml for each CF was calculated from 2-1.0 ml desiccated subsamples using a Denver Instruments balance. The amount of inoculum needed to treat at the targeted concentration was based on the mean dry bacterial cell mass/ml. The targeted treatment concentration was 50 ppm (dry bacterial mass/water volume) for flow-through pipes.
- Preparation of mussels: *Dreissena sp.* were collected from various locations, brought to the testing site, sieved, and stored until testing began. Collection sites included: the Mohawk River (Crescent, NY), Seneca Lake (Geneva, NY), Irondequoit Bay (Rochester, NY), and the research trailer at RG&E (Rochester, NY).
- Static treatment of mussels with CF: The day before the test, mussels were picked, placed into glass testing jars containing 100 ml of aerated hard water (Peltier and Weber, 1985) and allowed to attach overnight. The morning of testing, unattached mussels were replaced with attached mussels from an extra dish. At least one hour before treatment, the testing jars were filled with 495 ml of aerated hard water, set up with aeration, and labeled (Figs. 1 and 2). Mussels were exposed for the treatment period (24 hr), then the fluid was poured off and mussels were collected in clean plastic dishes with oxygenated hard water to be examined for mortality. Mortality was scored, and the mussels were held in the dishes for at least an additional 9 days, changing the water and scoring mortality each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

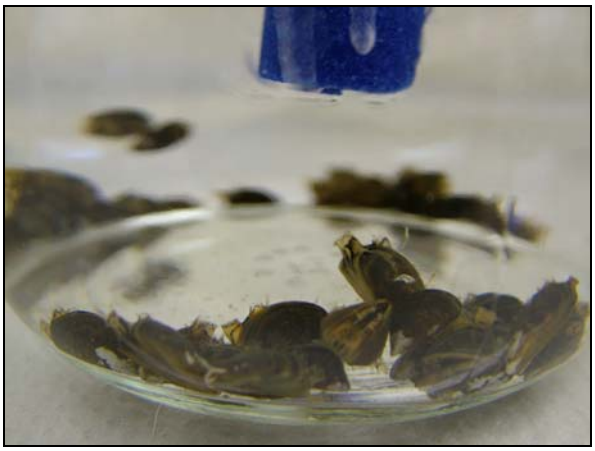

Figure 1. Zebra mussels shown siphoning in a control jar during experimentation.

Figure 2: Line of jars containing mussels being aerated during bacterial exposure.

- Flow-through treatment of mussels with CF: Several days before the test, mussels were picked, placed into acrylic pipes that were subsequently placed into a large flow-through tank and mussels allowed to attach within the pipes. The day before testing began, the pipes were connected to water flow by silicone tubing and flow-through conditions officially began. The flow-through apparatus in the Cambridge Laboratory is depicted in Figures 3 and 4. Mussels were exposed for the treatment period (24 hr), after which time the injection of bacterial cells was ended. Flow-through continued for another 5 days after which time the apparatus was disassembled and mortality checks began. Living mussels were collected in clean plastic dishes with oxygenated stream water to be examined for mortality. Mortality was scored. and the mussels were held in the dishes for an additional 20 days, changing the water and scoring mortality each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

Figure 3. Flow-through apparatus at the Cambridge Laboratory used for testing mussels in flowing stream water. The gray pump in the foreground controls water flow while the pump in the background controls inoculum injection. A similar apparatus was built for the RG&E research trailer.

Figure 4: Close-up of the flow-through pipes at Cambridge Laboratory during testing. The two shorter pipes are the control pipes, and the two longer and slightly cloudy pipes are the treatment pipes receiving approximately a 50-ppm concentration of *Pseudomonas fluorescens*-CL0145A cells.

2. CONFIRMING THE UNIQUE NATURE OF *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A.

The following is a general outline of the methodology employed in this test.

- Shaken seed cultures: 250-ml Erlenmeyer flasks containing 25 ml of bTSB were inoculated with 0.4 ml of glycerol stock culture containing each strain of *P. fluorescens*. Cultures were shaken at 200 rpm at 26±1ºC for 24 hr.
- Culturing static flask cultures in bTSB: 1 ml from the seed culture of each strain was used to inoculate each of 2 flasks, each containing 100 ml bTSB. Flasks were cultured statically for 72 hr at 26±1ºC.
- Culturing Bacterial Strain CL0145A: Shaken flask cultures: 0.35 ml from the seed culture of each strain was used to inoculate each of 3, each containing 35 ml ASM++. Flasks were cultured at a shaking speed of 200 rpm for 24 hr at 26±1ºC.
- Production of CF: The FWC from each flask was centrifuged and pellets from replicate flasks were combined to produce a single pellet for each strain for each test. FWC were centrifuged (20 min at 3400 rpm [Thermo Centra 8 Centrifuge]) in 50-ml centrifuge tubes, and cell pellets were re-suspended in a volume of dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \bullet 6H_2O$ in deionized water) to result in a dilution of ca. 10:1 (i.e., 10 ml FWC = 1 ml CF).
- Evaluation of Toxicity of CF: Mean dry bacterial cell mass/ml for CF was calculated from 2-0.5 ml desiccated subsamples using a Denver Instruments balance. The dry weight values were used to calculate the volume of each CF required to treat each of 3 replicate testing jars (3 replicate jars treated per each strain and culture medium) (500 ml synthetic hard water and 50 mussels) at a target concentration of 100 ppm (dry bacterial mass/water volume). Mussels were exposed for 24 hr.

- Preparation of zebra mussels: Mussels were collected from the Mohawk River near the Crescent Power Plant, brought back to the lab, sieved, and kept at 7ºC in unchlorinated tap water with filtration and aeration. Approximately 1 week before the test, mussels were removed from the cooler (7ºC) and acclimated to 23ºC in a 10-gal aquarium containing unchlorinated tap water with aeration (aquarium wrapped in towels to slow warming) and filtration. The day before the test, fifty 6-12 mm mussels were picked and placed in testing jars containing ca. 100 ml aerated hard water (creating a depth of ca. 3 cm) and allowed to attach overnight. The morning of the test, unattached mussels were removed and replaced with attached mussels from an extra dish and the water was replaced with 495 ml aerated hard water.
- Treatment of mussels with CF in aerated jars: Mussels were exposed for the treatment period (24 hr), then the fluid was poured off and mussels were collected in clean plastic dishes with oxygenated hard water to be examined for mortality. Mortality was scored and mussels were held in the dishes for an additional 9 days, changing the water and scoring mortality each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

RESULTS

This section gives an overview of the progress made during the six-month reporting period in both conducting and planning experiments.

1. DETERMINING THE EFFICACY OF *PSEUDOMONAS FLUORESCENS*-CL0145A HARVESTED FROM AN EXPERIMENTAL MEDIUM AGAINST QUAGGA AND ZEBRA MUSSELS UNDER FLOW THROUGH CONDITIONS.

In January 2005, *P. fluorescens*-strain CL0145A cells harvested from a recently developed experimental growth medium were tested against quagga mussels for the first time under aerated-jar and flow-through conditions. Prior testing, with cells harvested from a another "older" growth medium, indicated that while high levels of mortality could be achieved among treated zebra mussels, quagga mussels appeared much less susceptible. Cells harvested from the new experimental growth medium resulted in approximately 12 times more mortality among quagga mussel treatments when compared directly to mortality among quagga mussels treated with cells harvested from the older growth medium (78.67 $\pm$ 18.04% versus 6.67 $\pm$ 4.62%, respectively; Figure 5).

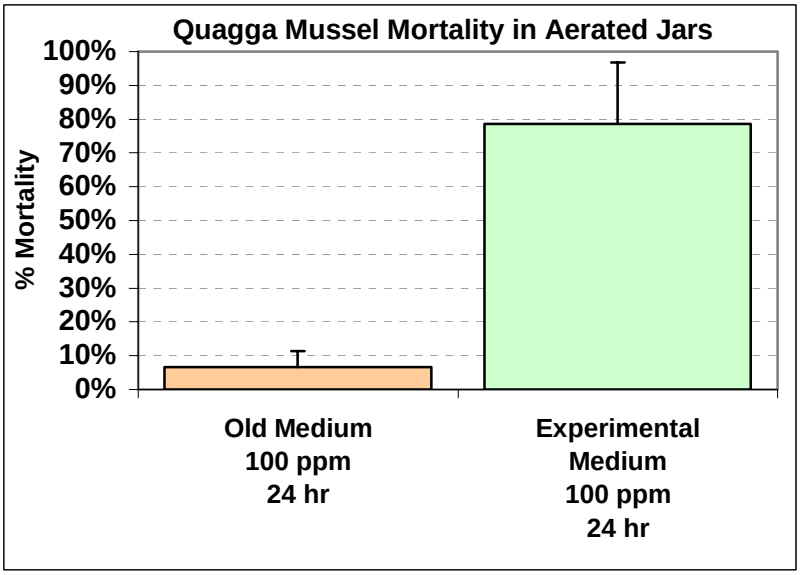

Figure 5: Comparison of mortalities between quagga mussels treated with *P. fluorescens*-CL0145A harvested from the "old" medium and the recently developed experimental medium. Treatment took place in aerated, glass testing jars. Mortality data were gathered for 10 days post-treatment.

A similar difference in quagga mussel mortalities was also apparent between the two growth media types when treatments occurred in stream water flow-through conditions as is indicated in Figure 6.

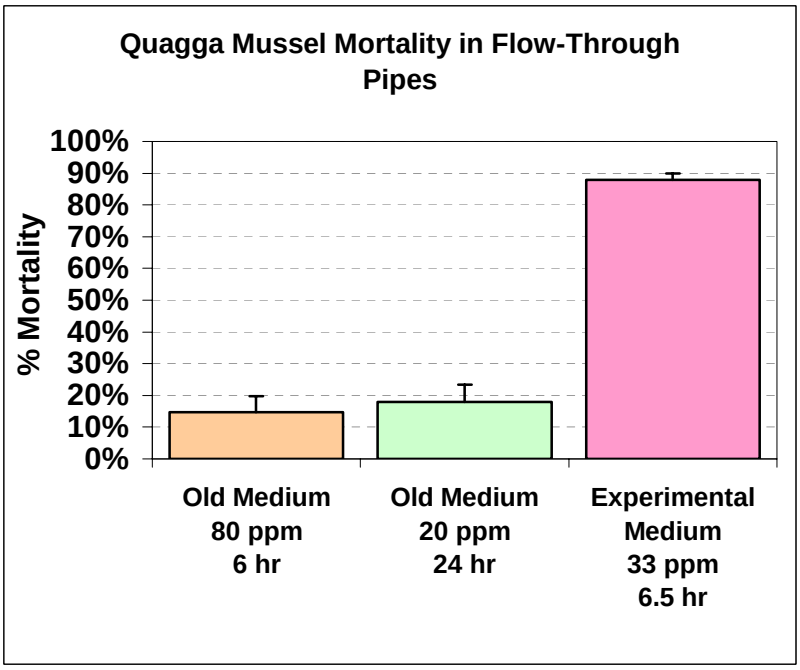

Figure 6: Seneca Lake quagga mussel mortality following treatment of stream water flow-through pipes with either *P. fluorescens*-CL0145A harvested from the "old" growth medium or the recently developed experimental medium. Mortality data were gathered for 48 days post-treatment.

The first step in the experimental approach to further testing the efficacy of *P. fluorescens*-CL0145A harvested from the new growth medium involved testing of local zebra and quagga mussels at the New York State Museum Field Research Laboratory located in Cambridge, NY under stream-water, flow-through conditions. In these Cambridge tests, quagga mussels from Seneca Lake (Geneva, NY) were assayed along with zebra mussels from the Mohawk River (Crescent, NY). The test was repeated the following week. Once this Cambridge testing was completed, two duplicate flow-through tests were carried out with *P. fluorescens*-CL0145A using quagga mussels collected from the RG&E Russell Power Station intake water system and zebra mussels from Irondequoit Bay (Rochester, NY). These latter duplicate trials were conducted inside the RG&E research trailer using the power plant's intake water. In both of these tests, the primary goal was to successfully treat both zebra and quagga mussels in flow-through pipes and achieve equally high levels of mortality against both species.

Cambridge Laboratory Efficacy Testing

Mohawk River zebra mussels and Seneca Lake quagga mussels that were used in the Cambridge Laboratory trials were similar in size (determined by measuring shell length). Mean shell lengths were: 7.3 ± 0.0 mm and 10.0 ± 1.6 mm, respectively (Table 1).

Table 1: Summary of mussel lengths from the two Cambridge flow-through trials.

Trial	Mussel Population ZM/QM	Mean (±SD) Length of Mussels Treated in Mini-Pipes		Combined Mean (±SD) ZM Length (mm)	Combined Mean (±SD) QM Length (mm)
		Zebra Mussels (mm)	Quagga Mussels (mm)		
1	Mohawk/Seneca	7.27 ± 1.41	11.19 ± 1.59	7.3 ± 0.0	10.0 ± 1.6
2	Mohawk/Seneca	7.27 ± 0.97	8.86 ± 1.89		

The mean mortalities achieved among both Mohawk River zebra mussels and Seneca Lake quagga mussels were similar when treated with *P. fluorescens*-CL0145A harvested from the experimental growth medium: 95.8% and 94.8%, respectively (Table 2, Figure 7).

Table 2: Mean mussel mortalities achieved following 24-hr treatment with *P. fluorescens*-CL0145A under controlled flow-through conditions at the Cambridge Laboratory.

Trial	Mussel Population ZM/QM	Effective Treatment Concentration (ppm)	25-day Mean % Mortality in Mini-Pipes (±SD)		Combined Mean (±SD) Mini-Pipe % Morality Data from 2 Tests	
			Zebra Mussels	Quagga Mussels	Zebra Mussels	Quagga Mussels
1	Mohawk/Seneca	31	94.9±2.9%	91.0±0.1%	95.8±2.2%	94.8±4.4%
2	Mohawk/Seneca	44	96.5±2.1%	98.5±0.7%		

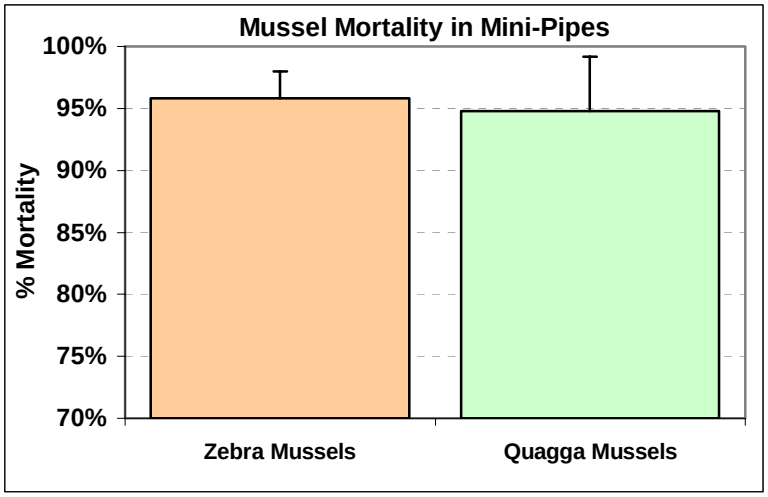

Figure 7: Mean mortalities achieved when treating mussels with *P. fluorescens*-CL0145A under controlled flow-through conditions at the Cambridge Laboratory.

RG&E Efficacy Testing

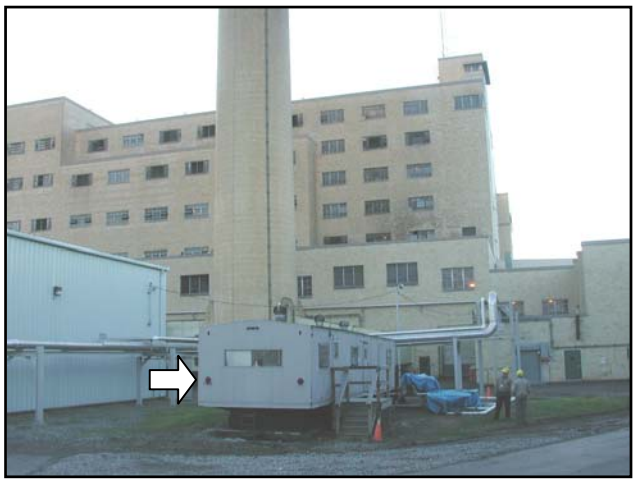

Figure. 8. The Rochester Gas & Electric Company has generously provided use of a research trailer with continuously-flowing intake water on the grounds of their Russell Power Station. The trailer contains areas for small-scale pipe tests as well as holding areas for maintaining mussels prior to conducting experiments.

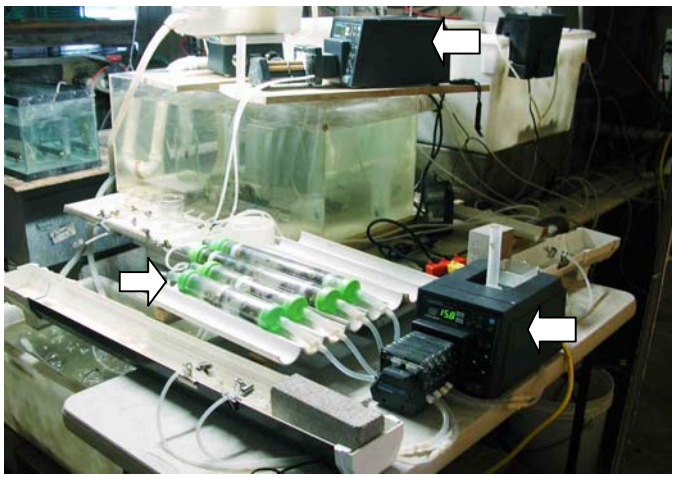

Figure 9. Within the trailer, small-scale tests are conducted using intake water diverted from the power plant. Acrylic pipes, such as the four pipes with green end caps in the foreground, hold mussels during experiments. The pump in the foreground controls water flow while the pump in the background injects bacteria.

Irondequoit Bay zebra mussels and RG&E quagga mussels tested in the RG&E research trailer trials were similar in size (determined by measuring shell length). Mean shell lengths were: 19.3 $\pm$ 1.9 mm and 19.8 $\pm$ 0.9 mm, respectively (Table 3).

Table 3: Summary of mussel lengths from the two RG&E flow-through trials.

Trial	Mussel Population ZM/QM	Mean Length (±SD) of Mussels Treated in Mini-Pipes		Combined Mean (±SD) ZM Length (mm)	Combined Mean (±SD) QM Length (mm)
		Zebra Mussels (mm)	Quagga Mussels (mm)		
1	Iron. Bay/RG&E	18.00 ± 4.00	20.42 ± 2.64	19.3 ± 1.9	19.8 ± 0.9
2	Iron. Bay/RG&E	20.65 ± 3.40	19.10 ± 3.33		

The mean mortalities achieved among both Irondequoit Bay zebra mussels and RG&E quagga mussels were similar when treated with *P. fluorescens*-CL0145A harvested from the experimental growth medium: 86.0% and 89.5%, respectively (Table 4, Figure 10).

Table 4: Mean mussel mortalities achieved following 24-hr treatment with *P. fluorescens*-CL0145A under controlled flow-through conditions in RG&E intake water.

Trial	Mussel Population ZM/QM	Effective Treatment Concentration (ppm)	25-day Mean % Mortality in Mini-Pipes (±SD)		Combined Mini-Pipe % Morality Data from 2 Tests	
			Zebra Mussels	Quagga Mussels	Zebra Mussels	Quagga Mussels
1	Iron. Bay/RG&E	46	84.0±5.7%	89.0±9.9%	86.0±6.3%	89.5±5.7%
2	Iron. Bay/RG&E	45	88.0±8.5%	90.0±0.0%		

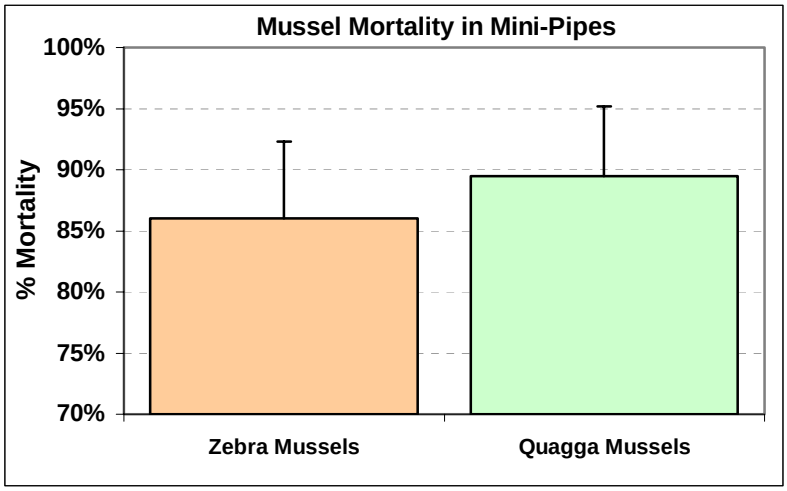

Figure 10: Mean mortalities achieved when treating mussels with *P. fluorescens*-CL0145A under controlled flow-through conditions in RG&E intake water.

2. CONFIRMING THE UNIQUE NATURE OF *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A.

Testing of *P. fluorescens*--CL0145A and the three currently-available sequenced strains of *P. fluorescens* (i.e., *Pf*-5, *Pf*0-1, and *Pf*-SBW25) were concurrently performed against zebra mussels from the Mohawk River in aerated jars. This test was performed twice, and the results

are shown in Table 5 and Figure 11. Treatment with *P. fluorescens*-CL0145A harvested from shaken, experimental growth medium, as expected, resulted in high levels of mortality among zebra mussels (mean kill >95%) while, in contrast, strains *Pf*-5, *Pf*0-1 and *Pf*-SBW25 appeared relatively non-toxic (e.g., mean mortality range: 1% to 11%).

Table 5: Toxicity of cells harvested from shaken experimental medium cultures of each *P. fluorescens* strain. Results after treating testing jars at 100 ppm for 24 hr (500 ml of treated hard water and 50 mussels).

***P. fluorescens* strain**	**Test #**	**% Mortality (20-day)**	**Mean % mortality (± SD)**
Pf-CL0145A	Test #1	90%, 96%, 100%	95.33±5.03%
	Test #2	100%, 100%, 92%	97.33±4.62%
Pf-5	Test #1	6%, 10%, 16%	10.67±5.03%
	Test #2	10%, 12%, 6%	9.37±2.99%
*Pf*0-1	Test #1	0%, 2%, 2%	1.33±1.15%
	Test #2	4%, 10%, 4%	6.00±3.46%
Pf-SBW25	Test #1	6%, 0%, 0%	2.00±3.46%
	Test #2	2%, 0%, 0%	0.67±1.15%

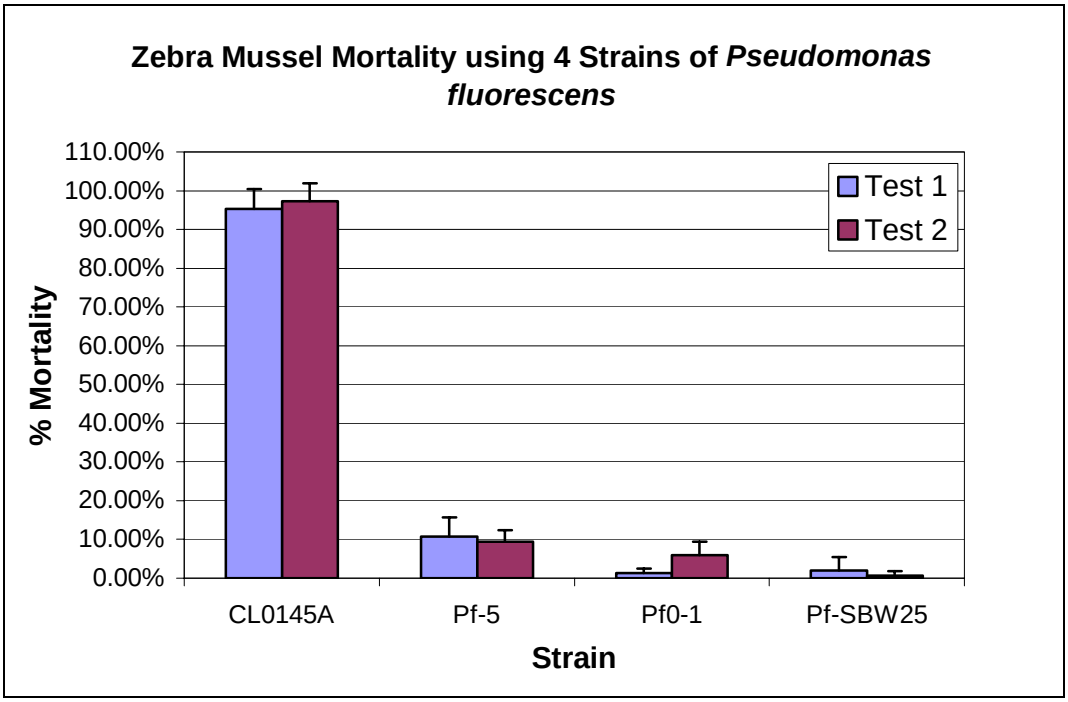

Figure 11: Mean (± SD) zebra mussel mortality for two tests assessing the efficacy of four strains of *Pseudomonas fluorescens*. Strain CL0145A was much more toxic to zebra mussels than any of the three sequenced *P. fluorescens* strains.

The choice of culturing method can have a major effect on the level of bacterial toxicity. Accordingly, these same four strains were grown up using another standard culturing method (i.e., static flasks of buffered tryptic soy broth) and assayed in two successive identical tests against zebra mussels from the Mohawk River in aerated jars (24 hr treatment at 100 ppm). Combining the data from these latter duplicate tests, the overall mean mussel mortality resulting form treatment with strains *Pf*-CL0145A, *Pf*-5, *Pf*0-1, and *Pf*-SBW25 were, respectively, 91.0%, 6.7%, 1.0%, and 2.3%. Thus, the results of these two additional tests closely matched those from the tests using cells produced using the more advanced shaken culturing medium. In summary, the cells of the three sequenced strains were not toxic when grown under the two different culturing methods.

DISCUSSION

1. DETERMINING THE EFFICACY OF *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A HARVESTED FROM AN EXPERIMENTAL MEDIUM AGAINST QUAGGA AND ZEBRA MUSSELS UNDER FLOW THROUGH CONDITIONS.

Coal-fired power plants within North America need an effective, economical, and non-polluting technique for managing infestations of the common zebra mussel and the quagga mussel (*Dreissena polymorpha* and *D. bugensis*, respectively) within their facilities. Due to a lack of options, many facilities have relied on the use of broad-spectrum, chemical biocides for control of these freshwater mussels. However, biocide treatments, such as continuous chlorination for three weeks, are widely regarded as environmentally unacceptable because they can result in the formation of potentially carcinogenic substances. Use of the bacterium *P. fluorescens*-CL0145A represents a potential alternative to the use of polluting biocide treatments and is the leading candidate in the world for the biological control of these macrofouling mussels. During our 2004 testing program, cells of *P. fluorescens* strain CL0145A that were harvested from a traditional growth medium consistently achieved relatively low levels of mortality among quagga mussels (*D. bugensis*). Quagga mussel mortality was often an order of magnitude lower than that of zebra mussels (*D. polymorpha*). If *P. fluorescens*-CL0145A is ever to become a commercially available biocontrol agent, it is essential that it be efficacious against zebra and quagga mussels, as over time the quagga mussel has slowly been replacing the common zebra mussel along its North American range. The data presented in this report have indicated that the choice of a culturing method is critically important in order for *P. fluorescens*-CL0145A to achieve high mortalities among both zebra and quagga mussels when treated in pipes under flow-through conditions. The potential market for *P. fluorescens*-CL0145A as a biocontrol agent will be much more extensive if, as a commercial product, it is efficacious against both the common zebra mussel and the quagga mussel.

One of the next research tasks will be to conduct a comprehensive series of flow-through trials in the RG&E research trailer in order to find a dosage (i.e., concentration and treatment duration) which can achieve >90% kill against *D. bugensis*, the less susceptible zebra mussel. Once completed, similar trials will then be carried out within the RG&E power station to confirm these results using actual plant service water.

2. CONFIRMING THE UNIQUE NATURE OF *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A.

Whole-genome sequencing of *P. fluorescens*-CL0145A would significantly increase the chances of its successful commercial development as a microbial pesticide for zebra mussel control. We now have characterized over 700 bacterial strains for zebra mussel toxicity, including 10 strains of *P. fluorescens*, and have established that among these bacterial strains only *P. fluorescens*-CL0145A is highly toxic to zebra mussels. Among the 9 nontoxic *P. fluorescens* strains that we have tested to date are the following three strains whose genomes have already been sequenced or are in the process of being sequenced: *Pf*-5, *Pf*0-1, and *Pf*-SBW25. The data in this report indicate that the latter three strains are relatively non-toxic to zebra mussels. These results are clearly very significant for this project because they further underscore the importance of sequencing *P. fluorescens*-CL0145A. In our effort to identify the natural product that is toxic to zebra mussels, the availability of genomic sequences from these latter three relatively nontoxic stains will greatly reduce the number of candidate genes which need to be tested. *P. fluorescens*-CL0145A genes with homology to genes in the latter three nontoxic strains will be disregarded at least initially, based on the hypothesis that they are not involved in zebra mussel toxicity. Following identification of the genes of interest, toxin production could be increased through site-directed mutagenesis and metabolic engineering, leading to a more effective commercial product. In addition, identifying the toxin would accelerate progress in a variety of research activities required for commercialization, such as: protocol improvements relating to bacterial fermentation, downstream processing, formulation, and product storage. At each step in these latter activities, the quantity of toxin could be monitored by relatively quick chemical analysis rather than by the more time-consuming, traditional approach of performing bioassays against zebra mussels. Although we have already made significant progress in devising improved culturing media and protocols to produce toxic cells, due to the low cost of currently used chemical biocides like chlorine, it will be very difficult to successfully commercialize *P. fluorescens*-CL0145A unless production costs can be considerably reduced. The primary avenue to achieve this is through genetic enhancement. That is why it is so critically important to have the whole genome of *P. fluorescens*-CL0145A sequenced. This would be the first step toward identifying the gene(s) involved in the production of the natural product that is lethal to zebra mussels. This is also expected to ultimately lead to increased toxin production per cell through the use of genetic and metabolic engineering techniques. We will resubmit a DOE-JGI-CSP proposal in 2006 for the whole genome sequencing of *P. fluorescens*-CL0145A. This resubmission should have an excellent chance for approval since it will include our data confirming that CL0145A is unique in its high toxicity to zebra mussels.

CONCLUSIONS

In the last six months of this project, significant progress has been made in tests designed to increase the efficacy of strain *P. fluorescens*-CL0145A against quagga mussels. Advances in culturing methods have lead to a new experimental growth medium which produces bacterial cells of significantly higher toxicity to quagga mussels.

It will be very difficult to successfully commercialize *P. fluorescens*-CL0145A unless production costs can be considerably reduced. The primary avenue to achieve this is through genetic enhancement of toxin production. Tests reported herein indicate that *P. fluorescens*-CL0145A has a uniquely high toxicity to zebra mussels when compared to the three other strains of *P.*

fluorescens that have already had their genomes sequenced. Our proposal for whole genome sequencing submitted to DOE-JGI-CSP in February 2005 was denied, largely due to lack of evidence that our strain was unique in its ability to kill zebra mussels. We plan to resubmit a DOE-JGI-CSP proposal in 2006 for the whole genome sequencing of *P. fluorescens*-CL0145A. This resubmission should have a good chance for approval since it will include our data confirming that CL0145A is unique in its high toxicity to zebra mussels.

REFERENCES

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This project was highlighted in the following presentation:

Molloy, D. P. Bacteria and zebra mussels: What's known about their interactions? March 17, 2005. Annual Meeting of the New England Association of Environmental Biologists, Lake George, NY. (Invited speaker.)